Volume 244, number 1, 217–222 FEB 06831 February 1989

Weak interaction of spectrin with phosphatidylcholine-phosphatidylserine multilayers: a ^{2}H and ^{31}P NMR study

Michel Bitbol*†, Christopher Dempsey†, Anthony Watts† and Phillippe F. Devaux

†*Department of Biochemistry, University of Oxford, Oxford OX1 3QU, England* and **Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France*

Received 8 November 1988; revised version received 24 December 1988

Spectrin from human erythrocytes binds to bilayer dispersions of both DMPC and DMPS:DMPC (1:1, w/w). However, no effect of bound spectrin on the conformation of the lipid head groups, as measured from the deuterium quadrupolar splittings of DMPC or DMPS specifically deuterated in the polar head groups, was detected in 1:1 mixtures of the two lipids containing either deuterated DMPC or DMPS. Neither the phase transition of the DMPS:DMPC mixtures, nor the spin-lattice relaxation time (T_1) of the deuterated DMPS head group, was affected by spectrin. These results argue against any strong interaction of spectrin with phosphatidylserine and rule out the possibility that spectrin is responsible for the maintainance of PS in the inner monolayer of the erythrocyte membrane during the whole life-span of this cell.

Spectrin; Phosphatidylserine; Phosphatidylcholine; NMR, ^{2}H-; NMR, ^{31}P-

1. INTRODUCTION

It has been proposed that the interaction between spectrin [1] and phosphatidylserine is sufficient to maintain the asymmetric distribution of phosphatidylserine across the erythrocyte membrane [2,3]. More recently, an ATP-dependent translocation of aminophospholipids from the outer to the inner leaflet of the erythrocyte membrane, catalysed by a specific membrane protein, has been demonstrated using spin-labeled probes [4–6] and confirmed by other methods [7–9]. The asymmetric distribution of aminophospholipids was accordingly accounted for by a dynamic equilibrium between outside-inside transport and inside-outside diffusion [10]. It was suggested that a strong spectrin-phosphatidylserine interaction might still play a role in slowing down the inside-outside diffusion of aminophospholipids which would otherwise be as fast as the outside-inside transport [11,12]. The involvement of spectrin in maintaining transmembrane phospholipid asymmetry was supported by evidence for a specific electrostatic binding between spectrin dimers and phosphatidylserine monolayers [13] or bilayers [14,15]. Despite evidence for a specificity in the interaction of spectrin with phosphatidylserine, a recent study has shown that the interaction energy is lower than the thermal energy at room temperature [17]. It has also been suggested that the affinity of spectrin for PS vesicles is not significantly different from its affinity for phosphatidylcholine vesicles [16]. In view of these disagreements, it seemed important to obtain new data about the possible specificity of PS-spectrin interaction. We report here a deuterium nuclear magnetic resonance study of the interaction between spectrin purified from human erythrocyte and bilayers of mixed dimyristoylphosphatidylserine (DMPS) and dimyristoylphosphatidylcholine (DMPC), where either DMPS or DMPC is deuterated in the polar head group.

Deuterium NMR has been shown to be a sensitive method to study the interaction between head group deuterated phospholipids, such as phosphatidylserine and a variety of membrane

Correspondence address: M. Bitbol, Department of Biochemistry, University of Oxford, Oxford OXI 3QU, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

proteins including peripheral membrane proteins [18], polymixin [19], cytochrome *c* [20], short charged artificial peptides [21] and bee venom melittin [22].

2. MATERIALS AND METHODS

2.1. *Spectrin*

Spectrin dimers from human erythrocytes were prepared as indicated in [23]. Their purity was confirmed by SDS-gel electrophoresis using the buffer system of Laemmli [24]. Spectrin was finally dialysed overnight against 100 mM Tris-HCl, 1 mM EDTA, pH 7.3.

2.2. *Lipids*

The head group deuterated phospholipid $\alpha\beta$-deuterated DMPS ($DMPSd_3$) was synthesized as described in [25], and $\alpha\beta$-deuterated DMPC ($DMPC\text{-}d_4$) and γ-deuterated DMPC ($DMPC\text{-}d_8$) were prepared as indicated in [26]. DMPC was purchased from Fluka. DMPS was prepared as indicated in [27]. All lipids were homogeneous by silica gel TLC.

2.3. *Sample preparation*

Samples for NMR experiments were prepared by mixing stock solutions of each lipid in chloroform/methanol/water (65:25:4, v/v) to give the desired composition. The solvent was removed in a stream of dry nitrogen at 40°C and the lipid mixtures dried under high vacuum overnight, and fully hydrated at 40°C with an excess of 100 mM Tris-HCl, 1 mM EDTA buffer, pH 7.3, containing various concentrations of spectrin dimers, from 0 to 1 mM. The lipid vesicles were ultracentrifuged (120000 × *g*; 50 min; 10°C) and the amount of bound protein was determined by measuring the absorbance of the supernatant at 280 nm. The pellet was then washed once with the same buffer prepared in deuterium-depleted water. Ultracentrifugation and measurement of the optical density of the supernatant were repeated to determine the final amount of bound protein. The final concentration of lipids in the pellet used for NMR measurements was 50–100 mg/ml.

2.4. *Binding experiments*

Dried lipid mixtures of either 0.5 mg DMPS + 0.5 mg DMPC, or 1 mg of pure DMPC were prepared as described above. These were hydrated and vortexed at 40°C with 1 ml of spectrin solution at various concentrations. The lipid suspensions were centrifuged (120000 × *g*; 50 min; 10°C) and the absorbance of the supernatant was measured at 280 nm to determine the amount of bound spectrin.

2.5. *^2H and ^{31}P NMR spectroscopy*

NMR spectra were recorded on a Nicolet 360 MHz spectrometer operating at 55.3 MHz for ^2H and at 145.1 MHz for ^{31}P. Single 90° pulses were used (14 μs for ^2H and 9 μs for ^{31}P). For ^2H NMR spectra, typically 20000–50000 transients were accumulated with a repetition time ranging between 50 (for $DMPS\text{-}d_3$) and 150 ms (for $DMPC\text{-}d_4/d_8$). Deuterium spin-lattice relaxation times (T_1) were measured by using an inversion-recovery pulse sequence. Temperatures were controlled with a thermostated gas flow unit and were accurate to within 2°C as determined by calibration against the pure DMPC phase transition.

3. RESULTS

3.1. *Binding experiments*

Fig.1 shows the amount of lipid-bound spectrin as a function of the total amount of protein in the suspension for either pure DMPC or DMPC:DMPS (1:1) suspensions. A binding constant $K_a \cong 10^7\ M^{-1}$ is obtained for both lipid preparations with no significant difference between them. The number of sites for PS or PC on each spectrin molecule is $n \cong 10^4$.

3.2. *^{31}P NMR experiments*

DMPC:DMPS (1:1) dispersions exhibit phosphorus-31 NMR spectra characteristic of partial membrane orientation (increased 90° orientational component of the powder pattern) (fig.2a). This spectrum contains a narrow line, which may be due to the presence of small vesicles. The addition of spectrin results in a phosphorus-31 powder pattern more characteristic of a spherical distribution of orientational components typical of unoriented bilayers (fig.2b).

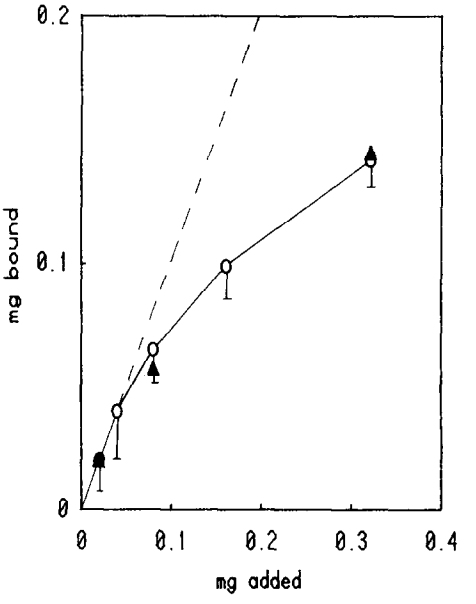

Fig.1. Binding of spectrin dimers to liposomes as a function of the amount of protein in the dispersion (1 ml volume). DMPC (1 mg) (▲); DMPS:DMPC (0.5:0.5 mg) (○).

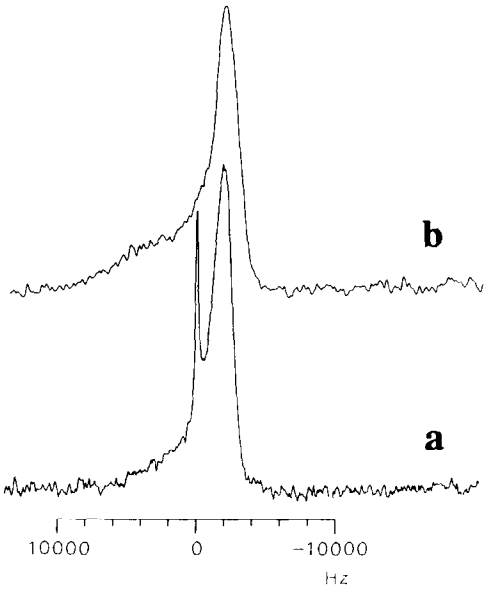

Fig.2. Phosphorus-31 NMR spectra of DMPC:DMPS dispersions (1:1, w/w; 20 mg total lipid in 0.3 ml; 35°C) either in the absence (a) or in the presence (b) of 4.5 mg of bound spectrin.

3.3. *^2H NMR experiments*

Deuterium NMR spectra at 35°C of head group deuterated DMPC mixed with equimolar DMPS are shown in fig.3. The head group deuterated DMPC is a mixture of DMPC-d_4 and DMPC-d_9 (9:1, w/w). The spectrum from the protein-free dispersions (fig.3a) shows three distinct quadrupolar splittings, the internal one (0.8 kHz) corresponding to the deuterons of the $N(CD_3)_3$ groups of DMPC-d_9, the intermediate splitting (2.4 kHz) from the deuterons in β-positions of DMPC-d_4, while the largest splittings partially resolved into two splittings (7.8 and 8.6 kHz) correspond to the two deuterons in α-position of DMPC-d_4 [28]. The magnitude of these splittings is unaffected by bound spectrin (fig.3b) although the spectral lines are narrower in the presence of spectrin.

Deuterium NMR spectra at 35°C of head group deuterated DMPS-d_3 mixed with DMPC (1:1, w/w) (fig.4a) show three resolved quadrupolar splittings, the two inner ones (2.8 and 9.7 kHz, respectively) corresponding to the deuterons in the α-position of the serine head group, and the larger one (14 kHz) corresponding to the deuteron in β-position [28]. Again, there is no observable effect of the incorporation of spectrin on the magnitude of these splittings (fig.4b).

However, alteration of lineshape is observed. Indeed, the spectrum of pure lipids displays sharp

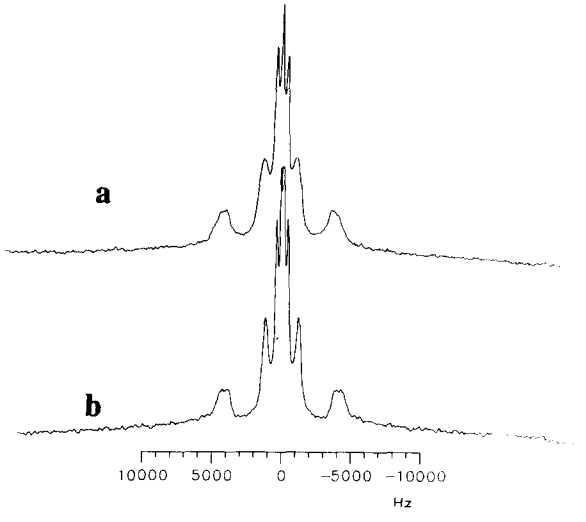

Fig.3. Deuterium NMR spectra of DMPC-d_g:DMPC-d_4:DMPS dispersions (1:9:10; w/w; 20 mg total lipid in 0.3 ml; 35°C) either in the absence (a) or in the presence (b) of 2.4 mg of bound spectrin.

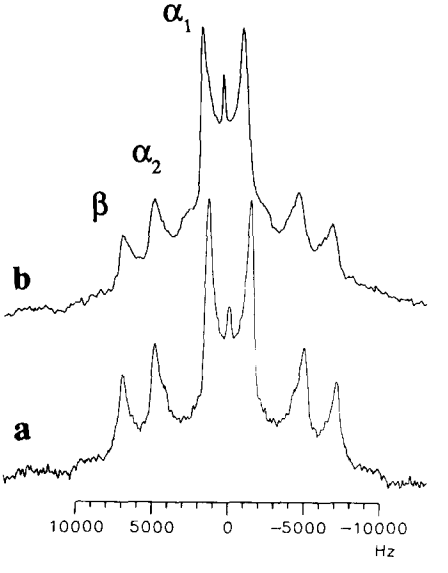

Fig.4. Deuterium NMR spectra of DMPC:DMPS-d_3 dispersions (1:1, w/w; 20 mg total lipid in 0.3 ml; 35°C) either in the absence (a) or in the presence (b) of 4.5 mg of bound spectrin.

edge lines and no observable external shoulders, whereas incorporation of spectrin yields shorter edges and enhanced shoulders. This result is in good agreement with ^{31}P experiments.

The temperature dependence of the deuterium quadrupole splittings of DMPS-d_3 in DMPC-DMPS-d_3 dispersions with and without spectrin (23 wt%) is shown in fig.5. The gel-liquid crystalline phase transition for these dispersions occurs at about 24°C for both pure lipid and spectrin-containing complexes. The deuterium spin-lattice relaxation times (T_1) for the DMPS-d_3 deuterons in these dispersions (6.7 ± 0.5 ms for the α-deuterons; 8.6 ± 0.5 ms for the β-deuteron at 35°C) were also unaffected by the presence of spectrin.

4. DISCUSSION

The lack of specificity in the binding of spectrin to phosphatidylserine compared with phosphatidylcholine (fig.1) is in agreement with previous work [13,16,29]. The high-binding constant (10^7 M^{-1}) is to be interpreted in view of the high number of sites per molecule of spectrin (10^4). This number itself is in reasonable agreement with measurements performed (on PS) by other methods [14]. The binding data also demonstrate that a large amount of spectrin (up to 23 wt% relative to lipid) is bound to the mixed lipid dispersions for which NMB data were obtained. It has been shown that the measured quadrupole splittings of head group deuterated phospholipids are sensitive to structural perturbations of the membrane surface [36,43,44]. Thus, the absence of any effect of bound spectrin on the quadrupolar splittings and T_1 relaxation times indicates that no significant change in the DMPS-d_3 conformation or dynamics of the DMPS head group are induced by the protein, even with a large amount of spectrin (23 wt% = 0.39×10^{-3} mol%) that is similar to the physiological spectrin-lipid ratio (10^{-3} mol%) [7] in the inner leaflet of the erythrocyte membrane. This result can be compared with other deuterium NMR studies of the interaction between PS and various proteins [20–22,30], which show that positively charged proteins which do not penetrate within the bilayer have detectable (though weak) effects on the conformation of PS head group [20,21]. Proteins which are both charged and able to penetrate into the membrane surface have a much larger effect on the conformation of PS head group [30]. The absence of any effect of spectrin on the conformation of PS head group suggests that the interaction of spectrin with these lipids is purely superficial and has no hydrophobic component, despite the presence of some hydrophobic segments on the protein [31]. Spectrin has an overall negative charge [32] above its isoelectric point of 5.6 [33]. Direct association with negatively charged lipids above pH 6.5 [2] may take place through orientation of positively charged segments of spectrin towards the polar moiety of the lipids [7].

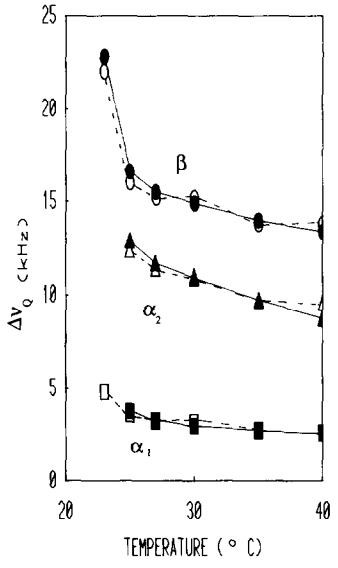

Fig.5. Quadrupole splittings from head group deuterated DMPS-d_3 in mixed bilayers with DMPC (1 : 1, w/w; 20 mg total lipid in 0.3 ml) as a function of temperature, in the absence (●, ▲, ■) and in the presence (○, △, □) of 4.5 mg of bound spectrin.

Due to the relatively narrow frequency band of the spectrometer, we cannot rule out the possibility that part of the signal was transformed into a broad undetectable spectral component from immobilized lipid head groups bound to spectrin. Such an immobilization is unlikely because no loss of spectral intensity was observed between protein-free and spectrin-containing complexes and also from the lack of effect of spectrin on the quadrupole splittings of head group deuterated DMPC (DMPC-d_4/d_8) in DMPC:DMPS mixtures. The DMPC head group splittings are sen-

sitive to the presence of DMPS [26] and any loss of DMPS from the mixed lipid phase by binding to spectrin would be expected to be reflected in a decreased PS-induced perturbation of the DMPC head group quadrupole splittings [19,22]. Moreover, it has been shown that fluorescent labeled phosphatidylserine has a faster lateral diffusion on the inner face of the erythrocyte membrane where spectrin is present, than on the outer face [35], with no detectable immobilized component.

The absence of any effect of spectrin on the quadrupolar splittings of DMPC deuterated in the head group, in 1:1 mixtures with DMPS, provides further evidence of the nature of spectrin/lipid interactions. The presence of charged lipids such as DMPS [28], of ions [36] or of charged anaesthetics [37] near the deuterated head group of DMPC strongly modifies its conformation, as a result of the perturbation of the bilayer surface charge [43]. Partial neutralization or screening of DMPS charges by positive charges at the membrane surface allows the DMPC head group to recover its original conformation [22]. Neutralization of the surface potential resulting from DMPS negative charges, by segmental positive charges on spectrin, thus seems to be precluded by our negative result. It is more likely that a dipolar interaction between spectrin and the lipid/water interface, occurs, and that this type of interaction accounts for the lack of specificity of phospholipid/spectrin binding which we report here. Such an interaction was previously ruled out, on the grounds that spectrin does not affect the phase transition of pure DMPC or DMPE monolayers, whereas it modifies the transition of pure DMPS or DMPS:DMPE 1:1 monolayers [7]. However, the temperature of the gel-liquid crystalline phase transition for zwitterionic lipids is determined by hydrophobic interactions between the acyl chains, while an additional electrostatic effect on the phase transition is observed when the lipids are charged [28]. A purely superficial dipolar interaction with the phospholipid polar heads accounting for the non-specific spectrin-lipid binding, would modify only the electrostatic component of the phase transition, and would thus be more efficient in changing the DMPS phase transition compared with that of DMPC or of a DMPS:DMPC 1:1 mixture. A selective effect of spectrin on the DMPS phase transition does not therefore imply a specificity of the mode of interaction between DMPS and spectrin.

The lineshapes of both phosphorus and deuterium spectra in pure lipid mixtures of DMPC:DMPS 1:1 do not correspond to what one would expect from an isotropically oriented bilayer. The 90° edge lines are enhanced, whereas the 0° shoulder has almost disappeared. Similar characteristics have been reported with other mixtures including charged lipids, such as POPE-POPG [38], and they were attributed to partial orientation of the bilayers in the magnetic field. It was otherwise suggested [21] that addition of a positively charged polypeptide could have a screening effect upon the negatively charged bilayers, leading to a partial dehydratation and to transition from unilamellar [39,40] to multilamellar liposomes. Here, the same effect was obtained with spectrin, a protein with overall negative charge.

The results of the present NMR study indicate that the interaction of spectrin with PS in mixed bilayers with zwitterionic lipid is weak and non-specific, supporting the conclusion that spectrin is unlikely to play a major role in maintaining phospholipid asymmetry in red blood cells. This is consistent with previous results which show that spectrin interacts only loosely with the cytoplasmic face of the erythrocyte membrane [41] and with the demonstration that heat-induced spectrin-depleted vesicles are able to establish and to maintain lipid asymmetry to the same extent as erythrocytes themselves [42].

Acknowledgements: We are grateful to the Oxford Enzyme group for the use of the Nicolet 360 MHz spectrometer and to Paulette Hervé and Peter Fisher for the synthesis of the deuterated lipids. This work was supported by grants from the Royal Society for an Exchange Fellowship (M.B.), and the CNRS (UA 526), the SERC for grants GR/D/6984 and GR/D/22889 to A.W. and the Wellcome Foundation for support of the NMR facility.

REFERENCES

[1] Bennett, V. (1985) Annu. Rev. Biochem. 54, 273–304.
[2] Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
[3] Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1980) in: Membrane Transport in Erythrocytes (Lassen, U.V. et al. eds) pp.108–119, Munksgaard, Copenhagen.

[4] Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
[5] Zachowski, A., Favre, E., Cribier, S., Hervé, P. and Devaux, P.F. (1986) Biochemistry 24, 5406–5416.
[6] Bitbol, M., Fellmann, P., Zachowski, A. and Devaux, P.F. (1987) Biochim. Biophys. Acta 904, 268–282.
[7] Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27.
[8] Connor, J. and Schroit, A.J. (1987) Biochemistry 26, 5099–5105.
[9] Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416.
[10] Bitbol, M. and Devaux, P.F. (1988) Proc. Natl. Acad. Sci. USA 85, 6783–6787.
[11] Williamson, P., Antia, R. and Schlegel, R.A. (1987) FEBS Lett. 219, 316–320.
[12] Middelkoop, E., Lubin, B.H., Bevers, E.M., Op den Kamp, J.A.F., Comfurius, P., Chiu, D.T.-Y., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1988) Biochim. Biophys. Acta 937, 281–288.
[13] Mombers, C., De Gier, J., Demel, R.A. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 603, 52–62.
[14] Bonnet, D. and Begard, E. (1984) Biochem. Biophys. Res. Commun. 120, 344–350.
[15] Cohen, A.M., Liu, S.C., Derick, L.H. and Palek, J. (1986) Blood 68, 920–926.
[16] Sikorski, A.F., Michalak, K. and Bobrowska, M. (1987) Biochim. Biophys. Acta 904, 55–60.
[17] Maksimiw, R., Sui, S.F., Gaub, H. and Sackmann, E. (1987) Biochemistry 26, 2983–2990.
[18] Sixl, F., Brophy, P.J. and Watts, A. (1984) Biochemistry 23, 2032–2039.
[19] Sixl, F. and Watts, A. (1985) Biochemistry 24, 7906–7910.
[20] Devaux, P.F., Hoatson, G.L., Favre, E., Fellmann, P., Farren, B., McKay, A.L. and Bloom, M. (1986) Biochemistry 25, 3804–3812.
[12] Roux, M., Neumann, L.M., Bloom, M. and Devaux, P.F. (1988) Eur. Biophys. J., in press.
[22] Dempsey, C., Bitbol, M. and Watts, A. (1988) Biochemistry, submitted.
[23] Gratzer, W.B. (1982) Methods Enzymol. 85, 475–481.
[24] Laemmli, U.K. (1970) Nature (London) 227, 680–687.
[25] Roux, M. and Neumann, J.M. (1986) FEBS Lett. 199, 33–38.
[26] Sixl, F. and Watts, A. (1982) Biochemistry 21, 6446–6452.
[27] Sixl, F. and Watts, A. (1983) Proc. Natl. Acad. Sci. USA 80, 1613–1615.
[28] Browning, J.L. and Seelig, J. (1980) Biochemistry 19, 1262–1270.
[29] Chang, C.H., Takeuchi, H., Ito, T., Machida, K. and Ohnishi, S. (1981) J. Biochem. 90, 997–1004.
[30] Roux, M., Neumann, J.M., Hodges, R.S., Devaux, P.F. and Bloom, M. (1988) Biochemistry, submitted.
[31] Calvert, R., Ungewickell, E. and Gratzer, W.B. (1985) Eur. J. Biochem. 107, 363–367.
[32] Speicher, D.W., Davis, G. and Marchesi, T. (1983) J. Biol. Chem. 258, 14938–14947.
[33] Hsu, C.J., Lemay, A., Eshdat, Y. and Marchesi, V.T. (1979) J. Supramol. Struct. 10, 227–239.
[34] Devaux, P.F. and Seigneuret, M. (1985) Biochim. Biophys. Acta 822, 63–125.
[35] Morrot, G., Cribier, S., Devaux, P.F., Geldwerth, D., Davoust, J., Bureau, Fellmann, P., Hervé, P. and Frilley, B. (1986) Proc. Natl. Acad. Sci. USA 83, 6863–6867.
[36] MacDonald, P.M. and Seelig, J. (1987) Biochemistry 26, 1231–1240.
[37] Boulanger, Y., Schreier, S. and Smith, J. (1981) Biochemistry 20, 6824–6830.
[38] Seelig, J., Borie, F. and Cross, T.A. (1985) Biochim. Biophys. Acta 814, 195–198.
[39] Atkinson, D., Hauser, H., Shipley, G.G. and Stubbs, J.M. (1974) Biochim. Biophys. Acta 339, 10–29.
[40] Speyer, J.B., Sripada, P.K., Das Gupta, S.K., Shipley, G.G. and Griffin, R.G. (1987) Biophys. J. 51, 687–691.
[41] Lemaigre-Dubreuil, Y. and Cassoly, R. (1985) Arch. Biochem. Biophys. 223, 495–502.
[42] Calvez, J.Y., Zachowski, A., Herrmann, A., Morrot, G. and Devaux, P.F. (1988) Biochemistry 27, 5666–5670.
[43] Seelig, J., MacDonald, P.M. and Scherer, S. (1987) Biochemistry 26, 7535–7541.
[44] Watts, A. (1987) Bioenerg. Biomembr. 19, 625–653.